TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		Attorney's Docket Number 046124-5042 U.S. Application No. 09/646785 Unassigned
International Application. No.	International Filing Date	Priority Date Claimed
PCT/JP99/01448	23 March 1999	24 March 1998

532 Rec'd PCT/PTC 22 SEP 2000

Title of Invention
VASCULARIZATION INHIBITORS
Applicants For DO/EO/US

Tadamitsu KISHIMOTO, Takashi NAGASAWA and Kazunobu TACHIBANA

Applicants herewith submit to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

[X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
[ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
[ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
[X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
[X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
  a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
  b. [X] has been transmitted by the International Bureau.
  c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
[X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
[X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)).
  a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
  b. [ ] have been transmitted by the International Bureau.
  c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
  d. [X] have not been made and will not be made.
[ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
[ ] An oath or declaration of the inventors (35 U.S.C. 371(c)(4)).
[ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

ems 11. to 14. below concern other document(s) or information included:

1. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
2. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
3. [X] A FIRST preliminary amendment.
   [ ] A SECOND or SUBSEQUENT preliminary amendment.
4. [X] Other items or information:
   a. [X] WIPO Publication WO99/48528 - (first page including abstract)
   b. [X] PCT/IB/304
   c. [X] PCT/IB/308
   d. [X] Computer readable copy of Sequence Listing
   e. [X] Statement Accompanying Sequence Listing

.S. APPLICATION NO.	INTERNATIONAL APPLICATION NO.	ATTORNEY DOCKET NUMBER
Unassigned 09/646785	PCT/JP99/01448	046124-5042

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5. [X] The following fees are submitted:

Basic National Fee (37 CFR 1.492(a)(1)-(5)):		
Search Report has been prepared by the EPO or JPO.....$840.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482).....$670.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)).....$760.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....$970.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....$96.00		
ENTER APPROPRIATE BASIC FEE AMOUNT	=	$ 840.00
urcharge of $130.00 for furnishing the oath or declaration later than] 20 [ ] 30 months from the earliest claimed priority date 37 CFR 1.492(e)).		$

Claims	Number Filed	Number Extra	Rate	
Total Claims	27 - 20 =	7	X $18.00	$126.00
ndependent Claims	8 - 3 =	5	X $78.00	$390.00
Multiple dependent claim(s) (if applicable)			+$260.00	$

TOTAL OF ABOVE CALCULATIONS	=	**$ 1,356.00**
Reduction by ½ for filing by small entity, if applicable. Verified Small Entity statement must also be filed. (Note 37 CFR 1.9, 1.27, 1.28)		-$
SUBTOTAL	=	**$ 1,356.00**
Processing fee of $130.00 for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date 37 CFR 1.492(f)).		+$
TOTAL NATIONAL FEE	=	**$ 1,356.00**
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet 37 CFR 3.28, 3.31). $40.00 per property		+
TOTAL FEES ENCLOSED	=	**$ 1,356.00**
Amount to be refunded		$
charged		$

a. [X] A check in the amount of $1356.00 to cover the above fees is enclosed.

b. [ ] Please charge my Deposit Account No. 50-0310 in the amount of $-0- to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] **Except** for issue fees payable under 37 C.F.R. §1.18, the Commissioner is hereby authorized by this paper to charge any additional fees during the entire pendency of this application including fees due under 37 CFR §1.16 and §1.17 which may be required, or credit any overpayment to Deposit Account No. 50-0310.

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Submitted: September 22, 2000

PATENT
ATTORNEY DOCKET NO.: 046124-5042

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of : Tadamitsu KISHIMOTO, et al.	
U.S. National Phase Application Filed : September 22, 2000	
U.S. Application No.: To Be Assigned	Group Art Unit: Unassigned
Date of National Stage Entry : Concurrently	Examiner: Unassigned
Based on PCT/JP99/01448 Filed : March 24, 1998	
For: VASCULARIZATION INHIBITORS	

Commissioner for Patents
Washington, D.C. 20231

Sir:

PRELIMINARY AMENDMENT

Prior to the examination of the above-identified application on the merits, please amend the application, without prejudice, as follows:

IN THE CLAIMS:

Claim 5, lines 1 and 2, change "any of claims 1-4" to --claim 1--;

Claim 5, line 2, delete "very"--;

Claim 6, lines 1 and 2, change "any of claims 1-4" to --Claim 1--

Claim 7, lines 1 and 2, change"any of claims 1-4" to --Claim 1--;

Claim 7, line 2, delete "very";

Claim 8, lines 1 and 2, change "any of claims 1-4" to --Claim 1--;

Claim 8, line 2, delete "very";

Claim 19, line 5, change "phosphoraipase" to --phospholipase--;

Claim 20, line 4, change "on cell" to --on a cell--;

Claim 21, line 3, delete "very";

Claim 22, lines 2 and 3 change "for the inhibition of" to -polynucleotide capable of inhibiting the--;

Claim 23, line 2, change "shows inhibition against" to --inhibits--;

Claim 24, line 2, change "using" to --administering--;

Claim 24, line 3, after "CXCR4" insert --to a mammal in need thereof--;

Claim 25, line 2, change "using" to --administering--;

Claim 25, line 3, after "CXCR4" insert --to a mammal in need thereof--;

Claim 26, line 3, change "using" to --administering--;

Claim 26, line 4, after "CXCR4" insert --to a mammal in need thereof--;

Claim 27, line 2, change "using" to --administering--;

Claim 27, line 3, after "CXCR4" insert --to a mammal in need thereof--.

REMARKS

The changes to the claims requested above have been made so as to eliminate multiple claim dependencies and to present claim language more conventional for practice in the United States. These changes do not introduce new matter, nor do they alter the subject matter presented and examined in the corresponding International Application.

Respectfully submitted,

MORGAN, LEWIS & BOCKIUS LLP

By: Elizabeth C. Weimar
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DESCRIPTION

VASCULARIZATION INHIBITORS

Technical Field

This invention relates to a novel vascularization inhibitor, an anti-solid cancer agent, and a therapeutic agent for a disease pathologically caused by neovascularization, each comprising a CXCR4 inhibitor as the effective ingredient. Further, the invention relates to a tissue-repairing agent comprising a CXCR4 potentiator as the effective ingredient.

Background Art

In the past, it has been known that when tumor cells invade out of the blood vessels, vascular endothelial cells rupture. It is also known that neovascularization is deeply involved in the proliferation and migration of cancer and that tumor cells produce and release a variety of neovascularization factors. Especially, neovascularization is considered crucial to the proliferation of solid tumors.

Therefore, a substance that inhibits the neovascularization has the potential to be an anticancer agent that is provided with a novel mode of action. For this reason, several types of neovascularization inhibitory substances such as

steroids and metabolic products of microorganisms have already been tested for use. ("Manual for Studies on Cancer Invasion and Metastasis," The Cancer Metastasis Society Ed., Kinhodo Publisher, 159-182 (1994)). However, it is strongly desired that novel neovascularization inhibitory substances with the action of more effectively inhibiting the proliferation and metastasis of cancers be discovered.

Disclosure of the Invention

This invention provides a vascularization inhibitor, an anti-solid cancer agent, or a therapeutic agent for a disease pathologically caused by neovascularization, each comprising an inhibitor of a chemokine receptor with the action of more effectively inhibiting the proliferation, invasion and metastasis of a cancer. Further, an object of the invention is to provide a tissue-repairing agent comprising as the effective ingredient, a potentiator of chemokine receptors.

Specifically, the present inventors pursued extensive research in order to solve the above-identified problems; and as a result, they have discovered that when knockout mice lacking in pre-B-cell growth stimulating factor/stromal-cell derived factor (hereafter referred to as "PBSF/SDF-1" or "SDF-1") which is a CXC chemokine as well as in CXCR4 which

is a chemokine receptor are created, the vascularization in the mice is suppressed, and namely, suppression of CXCR4 results in the suppression of vascularization. Such finding means that the chemokine receptor CXCR4 is essential for neovascularization.

Neovascularization of living tissues generally occurs through remodeling of the preexisting vascular system when they grow to perform their specific functions during development. Analyses of the mutant mice have determined that the molecules required by early vascular systems are largely receptor tyrosine kinases and their ligands. (Risau, w. Nature 386, 671-674 (1997); Folkman, J. & D'Amore, P. A. Cell 87, 1158-1155 (1996); and Lindahl, P., et al., science 277, 242-245 (1997)). However, substances responsible for vascularization during organogenesis have not yet been identified, because most of these mice die during early gestation before development of their tissues.

The structure of chemokine receptor CXCR4 according to this invention has already been known. (Bleul, C. C. et al., Nature 382,829-883 (1996); Oberlin, E. et al., Nature 382, 888-835 (1996); and Nagasawa, T. et al., Proc. Natl. Acad. Sci. USA 93, 14726-14729 (1996)). CXCR4 is a seven-transmembrane-spanning G-protein-coupled protein and a receptor for

PBSF/SDF-1 which is a CXC chemokine. The aforementioned factor is thought to be responsible for B-cell lymphopoiesis, bone marrow myelopoiesis and cardiac ventricular septum formation (Nagasawa, T. et al., Nature 382, 685-688 (1996)). CXCR4 also functions as a co-receptor for T-cell-line-tropic HIV-1 (Feng, Y. et al., Science 272, 872-877 (1996)). CXCR4 has further been reported to be expressed in cultured endothelial cells (Volin, M. V. et al., Biochem. Biophys. Res. Commun. 242, 46-53 (1998)).

In addition, the present inventors have discovered that the above-mentioned CXCR4 is expressed in developing vascular endothelial cells, and that mice lacking CXCR4 or its ligand PBSF/SDF-1 show defective formation of the large vessels being supplied to the gastrointestinal tract. Such finding means that the CXCR4 and PBSF/SDF-1 signaling systems are essential for the formation of median arteriovein supplying nutrient to the gastrointestinal tract. Furthermore, the present inventors have found that mice lacking CXCR4 are apt to die *in utero* just as seen in mice lacking PGSF/SDF-1. Such finding suggests that CXCR4 is the most critical, primary physiological receptor for PBSF/SDF-1.

Based on the foregoing observations by the present inventors, it is contemplated that substances

capable of inhibiting the action due to CXCR4 may inhibit vascularization and thus can be effective anticancer agents, since the vascularization is essential for the maintenance and enlargement of cancerous tissues.

It is likewise contemplated that substances capable of inhibiting CXCR4 can be therapeutic agents for the treatment of diseases involving neovascularization.

It is further contemplated that promotion of the action due to CXCR4 accelerates vascularization and thus can be a remedy for a disease where the vascularization is desired.

More specifically, as will be summarized below, this invention provides a vascularization inhibitor, an anti-solid tumor agent, or a therapeutic agent for a disease pathologically caused by neovascularization, each comprising as the effective ingredient, a substance that inhibits the action due to CXCR4. The invention also provides a tissue-repairing agent or the like comprising as the effective ingredient, a substance that potentiates the action due to CXCR4.

That is, this invention provides a vascularization inhibitor comprising a CXCR4 inhibitor as the effective ingredient.

Also, this invention provides an anti-solid cancer agent comprising a CXCR4 inhibitor as the effective ingredient.

Further, this invention provides a therapeutic agent for a disease pathologically caused by neovascularization, comprising a CXCR4 inhibitor as the effective ingredient.

Still further, the invention provides a tissue-repairing agent comprising a CXCR4 potentiator as the effective ingredient.

Because the formation of median or large arterioveins is essential for the maintenance and enlargement of a cancer tissue that exceeds a certain size, the vascularization inhibitor of this invention blocks the CXCR4 or PBSF/SDF-1 signaling system, thus suppressing the maintenance and enlargement of the cancer tissue.

The finding obtained in this invention suggests the possibility that the CXCR4 and PBSF/SDF-1 signaling systems contribute to the universal vascularization. Therefore, in diseases of which a particular kind of cancer or neovascularization is the major pathological cause, it is likely that CXCR4 or PBSF/SDF-1 is deeply involved in the pathological cause; in this case, there is the possibility that these diseases can be suppressed by blocking CXCR4 or

PBSF/SDF-1 individually or concurrently with other molecules.

In the present specification and the drawings, the abbreviations for bases or amino acids are those following the IUPAC-IUB Commission on Biochemistry Nomenclature or those based on what is customary in the art. Illustrated below are their examples. Where amino acids are meant and there may be their optical isomers, they represent L-forms unless otherwise indicated.

DNA: deoxyribonucleic acid

cDNA: complementary deoxyribonucleic acid

A: adenine

T: thymine

G: guanine

C: cytosine

RNA: ribonucleic acid

mRNA: messenger ribonucleic acid

G or Gly: glycine

A or Ala: alanine

V or Val: valine

L or Leu: leucine

I or Ile: isoleucine

S or Ser: serine

T or Thr: threonine

C or Cys: cysteine

M or Met: methionine

E or Glu: glutamic acid

D or Asp: aspartic acid

K or Lys: lysine

R or Arg: arginine

H or His: histidine

F or Phe: phenylalanine

Y or Tyr: tyrosine

W or Trp: tryptophan

P or Pro: proline

N or Asn: asparagine

Q or Gln: glutamine

BSA: bovine serum albumin

FBS: fetal bovine serum

PBS: phosphate buffer saline

SDS: sodium dodecyl sulfate

Brief Description of the Drawings

Fig. 1 is a graph showing a targeting strategy for the CXCR4 gene. In the figure, there are shown the CXCR4 wild-type allele at the top, a targeting vector in the middle, and a predicted mutant allele at the bottom. The coding regions of the genes are indicated by black boxes. Empty boxes indicate the 5'- and 3'-untranslated regions. Dotted lines

indicate homologous fragments used in the targeting vector. Probe A is an external probe for Southern hybridization. Restriction sites are E (EcoRI), Sh (SphI), and X (XhoI), respectively.

Fig. 2A is a photograph showing the Southern blot analysis of tail DNAs from wild-type (+/+) and heterozygous mutant (+/-) mice. The EcoRI-EcoRI fragments from the 11.8-kb wild-type and the 8.2-kb targeted allele which were identified by probe A are shown in the figure.

Fig. 2B is a photograph showing the RT-PCR amplification analysis of CXCR4 expression. Total RNAs were prepared from E18.5 wild-type and homozygous mutant embryos, and amplified with CXCR4-specific primers. The RT-PCR amplification employed G3PDH mRNA, which was universally expressed, as a control for the presence of any amplifiable RNA.

Fig. 3 is a photograph showing defects of gastrointestinal blood vessels at the mesentery and mid-gut loop region in a wild-type $CXCR4^{-/-}$ embryo at E13.5, resulting from immunohistostaining of the mesentery and intestine with anti-PECAM-1 antibody. Arrow indicates a large branch of superior mesenteric artery or superior mesenteric vein being supplied to the small intestine in the wild-type mesentery. "du"

represents duodenum; "p," the proximal part of mid-gut loop; and "dm," the distal part of mid-gut loop.

Fig. 4 is a photograph showing defects of gastrointestinal blood vessels at the cross-sections of mesentery in the wild-type CXCR4$^{-/-}$ embryo at E13.5, resulting from immunohistostaining of the mesentery and intestine with the anti-PECAM-1 antibody. "a" represents artery, and "v" vein.

Fig. 5 is a photograph showing defects of gastrointestinal blood vessels in the jejunum in a wild-type CXCR4$^{-/-}$ embryo at E17.5, resulting from immunohistostaining of the mesentery and intestine with the anti-PECAM-1 antibody. Arrow indicates a large branch of superior mesenteric artery or superior mesenteric vein being supplied to the small intestine in the wild-type mesentery.

Fig. 6 is a photograph showing defects of gastrointestinal blood vessels at the more distal part of the jejunum in the wild-type CXCR4$^{-/-}$ embryo at E17.5, resulting from immunohistostaining of the mesentery and intestine with the anti-PECAM-1 antibody. Arrow indicates a large branch of superior mesenteric artery or superior mesenteric vein being supplied to the small intestine in the wild-type mesentery.

Fig. 7 is a photograph showing defects of gastrointestinal blood vessels at the mesentery and

mid-gut loop regions in a mutant CXCR4$^{-/-}$ embryo at E13.5, resulting from immunohistostaining of the mesentery and intestine of mutant with the anti-PECAM-1 antibody. "p" represents the proximal part of mid-gut loop; and "dm," the distal part of mid-gut loop.

Fig. 8 is a photograph showing defects of gastrointestinal blood vessels at the cross-sections of mesentery in the mutant CXCR4$^{-/-}$ embryo at E13.5, resulting from immunohistostaining of the mesentery and intestine of the mutant with the anti-PECAM-1 antibody.

Fig. 9 is a photograph showing defects of gastrointestinal blood vessels in the jejunum in a mutant CXCR4$^{-/-}$ embryo at E17.5, resulting from immunohistostaining of the mesentery and intestine of the mutant with the anti-PECAM-1 antibody.

Fig. 10 is a photograph showing defects of gastrointestinal blood vessels at a more distal part of the jejunum in the mutant CXCR4$^{-/-}$ embryo at 17.5, resulting from immunohistostaining of the mesentery and intestine of the mutant with the anti-PECAM-1 antibody.

Fig. 11 is a photograph showing defects of gastrointestinal blood vessels, which are a haemorrhagic lesion of the unstained intestine of a mutant mouse, an E16.5 mutant CXCR4$^{-/-}$ embryo,

resulting from immunohistostaining of the mesentery and intestine of the mutant with the anti-PECAM-1 antibody.

Fig. 12A is a photograph showing the result of immunohistostaining the stomach of an E13.5 wild-type with the anti-PECAM-1 antibody. Arrow indicates a large vessel only seen in the wild-type.

Fig. 12B is a photograph showing the result of immunohistostaining the stomach of an E13.5 mutant with the anti-PECAM-1 antibody.

Fig. 12C is a photograph showing the result of immunohistostaining the stomach of an E15.5 wild-type with the anti-PECAM-1 antibody. Inset in the photograph shows haematoxylin and eosin-stained sections of large vessels in the wall of stained stomach at E15.5. Arrow indicates a large vessel only seen in the wild-type.

Fig. 12D is a photograph showing the result of immunohistostaining the stomach of an E15.5 mutant with the anti-PECAM-1 antibody.

Fig. 13A is a photograph showing CXCR4 and PBSF/SDF-1 expression in a gastrointestinal tract tissue through *in situ* hybridization. Serial sections of the wild-type mesentery connecting to the mid-gut loop were stained with haematoxylin and eosin. "m"

represents mesentery, "i" intestine, "a" superior mesenteric artery, and "v" superior mesenteric vein.

Fig. 13B is a photograph showing CXCR4 and PBSF/SDF-1 expression in a gastrointestinal tract tissue through *in situ* hybridization. Hybridization was done with a CXCR4-specific probe. Arrows indicate the stained endothelial cells of the mesenteric vessels.

Fig. 13C is a photograph showing CXCR4 and PBSF/SDF-1 expression in a gastrointestinal tract tissue through *in situ* hybridization. Hybridization was done with a PBSF/SDF-1-specific probe. PBSF/SDF-1 was expressed in mesenchymal cells surrounding the endothelial cells in the mesentery.

Fig. 13D is a photograph showing CXCR4 and PBSF/SDF-1 expression in the gastrointestinal tract tissue through *in situ* hybridization. Serial sections of the wild-type mesentery connecting to the mid-gut loop were stained with haematoxylin and eosin. Fig. 13D is an enlargement of blood vessels arising from the superior mesenteric artery shown in Fig. 13A, where strong expression of CXCR4 was observed in the vascular endothelial cells.

Fig. 13E is a photograph showing CXCR4 and PBSF/SDF-1 expression in the gastrointestinal tract tissue through *in situ* hybridization. Hybridization

was done with the CXCR4-specific probe. Fig. 13E is an enlargement of blood vessels arising from the superior mesenteric artery shown in Fig. 13B, where strong expression of CXCR4 was observed in the vascular endothelial cells. Arrow indicates the stained endothelial cells of the mesenteric vessels.

Fig. 13F is a photograph showing CXCR4 and PBSF/SDF-1 expression in a gastrointestinal tract tissue through *in situ* hybridization. It is a section of an E18.5 wild-type embryonic bone marrow, showing CXCR4 expression in hematopoietic cells but no expression in spindle-shaped stroma cells.

Best Mode for Carrying out the Invention

The vascularization inhibitor, the anti-solid cancer agent, or the therapeutic agent for a disease pathologically caused by neovascularization according to this invention comprises as the effective ingredient, a substance that inhibits the action of CXCR4 which is a chemokine receptor. On the other hand, the tissue-repairing agent according to the invention comprises as the effective ingredient, a substance that potentiates the action of CXCR4.

The amino acid sequence of CXCR4 has already been known. Specifically, the amino acid sequence of human CXCR4 and the amino acid sequence of murine CXCR4 are set forth in SEQ ID NOs: 1 and 3,

respectively. The base sequence of human CXCR4 and the base sequence of murine CXCR4 are set forth in SEQ ID NO: 2 (base positions 1-1056) and SEQ ID NO: 4 (base positions 1-1077), respectively.

Also, the amino acid sequence of SDF-1, which is a ligand binding to CXCR4, has already been known. There are two types of SDF-1 differing in the length of amino acid sequence, i.e., SDF-1-α and SDF-1-β. Specifically, the amino acid sequence of human SDF-1-α is set forth in SEQ ID NO: 5 and its base sequence in SEQ ID NO: 6 (base positions 474-740). Human SDF-1-β (SEQ ID No: 9) is derived from human SDF-1-α by appending four amino acid residues, Arg Phe Lys Met, to a C-terminus thereof.

The amino acid sequence of murine SDF-1-α is set forth in SEQ ID NO: 7 and its base sequence in SEQ ID NO: 8 (base positions 82-348). Murine SDF-1-β (SEQ ID No: 10) is derived from murine SDF-1-α by appending four amino acid residues, Arg Leu Lys Met, to a C-terminus thereof. For human and murine SDF-1's, the sequence of from the 1st amino acid (Met) to the 21st amino acid (Gly) is a signal sequence.

CXC chemokines that have hitherto been known include, in addition to PBSF/SDF-1 mentioned above, IL-8 (Yoshimura., T. et al., Proc. Natl. Acad. Sci. U.S.A., 84, 9233-9237 (1987)), NAP-2 (Walz. A., et al.,

Biochem. Biophys. Res. Comun., 159, 969-975 (1989)), NAP-4, GRO α (Richmondo, A. et al., J. Cell. Biochem., 36, 185-198 (1988)), GRO β (Haskill, S. et al., Proc. Natl. Acad. Sci. U.S.A., 87, 77732-7736 (1990)), GRO γ (Haskill, S. et al., ibid.(1990)), GCP-2 (Proost, P. et al., J. Immunol., 150, 1000-1010 (1993)), ENA-78 (Wayz, A. et al., J. Exp. Med., 174, 1355-1362 (1991)), PF-4 (Deuel, T. F. et al., Proc. Natl. Acad. Sci. U.S.A. 74, 2256-2258 (1977)), and IP-10 (Dewald, B. et al., Immunol. Lett., 32, 81-84 (1992)).

There are no particular limitations to substances that inhibit the action due to CXCR4 that can be used in this invention; and they may be substances that inhibit the action due to CXCR4 with the result of inhibition of neovascularization.

Specifically mentioned are: (1) a substance based on inhibition of the binding itself between the ligand (SDF-1) and the receptor (CXCR4); (2) a substance based on inhibition of the signaling from CXCR4 to nuclei; (3) a substance that inhibits the expression of CXCR4 itself; and (4) a substance that inhibits the expression of SDF-1 itself.

(1) For the substance that inhibits the binding itself between SDF-1 and CXCR4, there are a substance that inhibits SDF-1 and a substance that inhibits CXCR4.

More specifically, the substance that inhibits SDF-1 is classified into a substance that inhibits CXCR4 in antagonistic competition with SDF-1 and a substance that inhibits SDF-1 from binding to CXCR4 by binding to SDF-1. For the substance that inhibits CXCR4 in antagonistic competition with SDF-1, there are concretely mentioned a protein having a SDF-1-like structure, a fused protein of the foregoing protein with another peptide or polypeptide, a low molecular weight compound having a structure similar to a partial peptide of SDF-1 or a binding site of SDF-1, and the like.

For the substance that inhibits SDF-1 from binding to CXCR4 by binding to SDF-1, there are concretely mentioned an anti-SDF-1 antibody, a fragment thereof having possessing binding activity, a fused protein possessing binding activity to SDF-1, a substance that induces a structural change in SDF-1, a low molecular weight compound that binds to the CXCR4-binding site of SDF-1, and the like.

More specifically, the substance that inhibits CXCR4 is classified into a substance that inhibits CXCR4 in antagonistic competition with CXCR4 for binding to SDF-1 and a substance that inhibits SDF-1 from binding to CXCR4 by binding to CXCR4. For the substance that inhibits CXCR4 in antagonistic

competition with CXCR4 for binding to SDF-1, there are concretely mentioned a soluble CXCR4 that antagonizes CXCR4 in inhibition, a protein having a CXCR4-like structure, a fused protein of the foregoing protein with another peptide or polypeptide, a low molecular weight compound having a structure similar to a partial peptide of CXCR4 or a binding site of CXCR4, and the like.

For the substance that inhibits SDF-1 from binding to CXCR4 by binding to CXCR4, there are concretely mentioned an anti-CXCR4 antibody, a fragment thereof possessing its binding activity, a fused protein possessing binding activity to CXCR4, a substance that induces a structural change in SDF-1, a low molecular weight compound that binds to the SDF-1-binding site, and the like.

Examples of the substance that inhibits the binding itself between CXCR4 and SDF-1 include T22 (T. Murakami, et al., J. Exp. Med., 186, 1389-1393 (1997)), ALX40-4C (J. Exp. Med., 186, 1395-1400 (1997)), AMD3100 (J. Exp. Med., 186, 1383-1388 (1997); Nat. Med., 4, 72-77 (1998)), and the like. As to the methods for preparation of these substances, they can, for example, be done by the method as described in J. Exp. Med., 186, 1189-1191 (1997) with any possible modifications.

(2) There is no particular limitation to the substance based on inhibition of the signaling from CXCR4 to nuclei insofar as it is a substance having such action. For the substance based on inhibition of the signaling from CXCR4 to nuclei, there are mentioned inhibitors of the signaling system existing downstream of a G protein-coupled protein, such as an MAK cascade inhibitor, a phospholipase C (PLC) inhibitor and a kinase inhibitor for PI3 kinase.

(3) For the substance that inhibits the expression of CXCR4 itself, there are mentioned a substance that apparently makes CXCR4 disappear on cells and a substance that inhibits the expression of CXCR4 itself. A specific example of the substance that apparently makes CXCR4 disappear on cells is a substance that induces down-regulation of CXCR4. The "induction of down-regulation of CXCR4" specifically means such a function that it acts on the cell membrane to alter mobility thereof and thereby to make CXCR4 disappear from the cell membrane. For example, dexamethasone is mentioned as a substance possessing the function.

For the substance that inhibits the expression of CXCR4 itself, there are concretely mentioned an antigene, an antisense (antisense oligonucleotide and antisense RNA expressed by antisense vector), a

ribozyme, and a substance that inhibit the expression control site of CXCR4 such as a promoter or an enhancer.

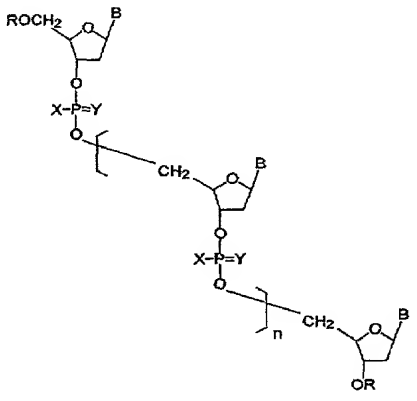
From the examples which will be described later, it has become evident that when a vector containing a part of the CXCR4 gene is used to cause the deficiency of CXCR4, vascularization is suppressed. Therefore, the inhibition of CXCR4 by the antigene, antisense, or ribozyme of CXCR4 will suppress vascularization.

Antisense oligonucleotides that can preferably be used in this invention include CXCR4 genes, SDF-1 genes against CXCR4, nucleotides (DNAs or RNAs) selectively hybridizable to the genes of substances that are involved in the signaling system based on CXCR4, and derivatives thereof (such as antisense oligonucleotides). This invention, for example, encompasses antisense oligonucleotides that hybridize to any site of the base sequence of human CXCR4 gene as set forth in SEQ ID NO: 2.

Preferably, the antisense oligonucleotide is an antisense oligonucleotide to at least 20 consecutive nucleotides within the base sequence set forth in SEQ ID NO: 2. More preferably, the antisense oligonucleotide is the at least 20 consecutive nucleotides containing a translation initiation codon.

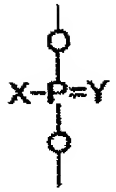
As used herein, "antisense oligonuclotide" is not only one having nucleotides that correspond to the nucleotides constituting the predetermined region of DNA or RNA and that are all complementary thereto, but also may allow one or more mismatches of nucleotide to be present therein insofar as the oligonuclotide and the DNA or the RNA are able to selectively and stably hybridize to the base sequence set forth in SEQ ID NO: 2. By "selectively and stably hybridize" is meant those having at least 70%, preferably at least 80%, more preferably at least 90%, most preferably 95% or greater homology of base sequence in the nucleotide sequence region of at least 20, and preferably 30 consecutive nucleotides. In the present specification, "homology" indicates "identity."

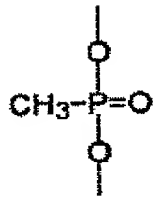
When the oligonucleotide derivative used in this invention is a deoxyribonucleotide, the structure of each derivative is represented by formula 1:

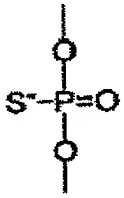


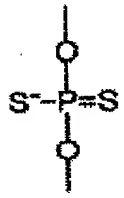
In the formula, X may independently be any of oxygen (O), sulfur (S), a lower alkyl group, a primary amine and a secondary amine. Y may independently be either oxygen (O) or sulfur (S). B is selected from adenine, guanine, thymine, or cytosine, and is principally an oligonucelotide complementary to DNA or RNA of the human CXCR4 gene. R is independently hydrogen(H), a dimethoxytrytyl group or a lower alkyl group. n is from 7 to 28.

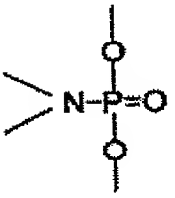
Preferable oligonucleotide derivatives are not limited to oligonucleotides that have not been modified, but may be modified oligonucleotides, as will be illustrated below. These modified forms include lower alkyl phosphonate derivatives of such types as methyl phosphonate or ethyl phosphonate, phosphorothioate derivatives, phosphoroamidates, and the like.

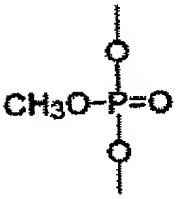
EXAMPLES OF: 

METHYL PHOSPHONATE: 

PHOSPHOROTHIOATE: 

PHOSPHORODITHIOATE: 

PHOSPHOROAMIDATE: 

PHOSPHORIC TRIESTER: 

These oligonucleotide derivatives can be obtained by standard methods as described below. The oligonucleotides of formula (1) wherein X and Y are both O may readily be prepared with a commercial DNA synthesizer such as one available from Applied Biosystems; for their preparation method, solid phase synthesis employing phosphoroamidites, solid phase synthesis employing hydrogen phosphonates and the like can be used to obtain them.

The phosphoric triester modified forms wherein X is a lower alkoxy group can be obtained by standard methods: for example, oligonucleotides obtained by chemical synthesis are treated with a DMF/methanol/2,6-lutidine solution of tosyl chloride. The alkyl phosphonate modified forms wherein X is an alkyl group can be obtained according standard methods, for example, by using phosphoamidites. The phosphorothioate modified forms wherein X is S can be obtained according standard methods, for example, by solid phase synthesis using sulfur, or alternatively by solid phase synthesis using tetraethyl thiuram disulfide. The phosphorodithioate modified forms wherein X and Y are both S can be obtained according to solid phase synthesis, for example, by converting bisamidites into thioamidites and allowing sulfur to act on the thioamidites. The phosphoroamidate

modified forms wherein X is a primary or secondary amine can be obtained according to solid phase synthesis, for example, by treating hydrogenphosphonates with a primary or secondary amine; alternatively, they can be obtained by oxidizing amidites with tert-butyl hydrogen peroxide.

Purification and the assurance of purity may be carried out by high speed liquid chromatography or polyacrylamide gel electrophoresis. The confirmation of molecular weights may be carried out by electrospray ionization mass spectrometry or fast atom bombardment-mass spectrometry.

The antisense oligonucleotide derivatives used in this invention act on a CXCR4 receptor or a ligand thereof, as well as on cells producing a signaling substance based on CXCR4, and bind to DNA or RNA encoding the peptide, thereby inhibiting its transcription or translation, or promoting the decomposition of mRNA; as a result, they possess an inhibitory effect on the action due to CXCR4 by suppressing the expression of the peptide.

Consequently, the antisense oligonucleotides used in this invention have utility in inhibiting neovascularization. Neovascularization inhibitors comprising the antisense oligonucleotides of this

invention are useful as therapeutic agents for cancers, particularly solid cancers.

Preparation of CXCR4 antisense vectors may follow the methods that are commonly used. Specifically, cDNA encoding CXCR4 is linked to an AAV vector (adeno-associated virus vector), MLV vector (murine leukemic virus vector), HIV vector, or the like in the antisense direction. By "antisense direction" is meant linking to the 3'-side of the cDNA to be introduced in the downstream of the promoter. The antisense RNAs synthesized from cDNAs contained in these vectors constitutively suppress the expression of CXCR4 in hosts.

Antisense DNAs or RNAs can be introduced into cells by using means such as the liposome method, the HVJ liposome method, or the positively charged liposome method. The introduction of a CXCR4 antisense DNA or RNA allows for the constitutive inhibition of expression of CXCR4.

(4) For the substance that inhibits the expression of SDF-1 itself against CXCR4, there are mentioned an antisense substance that inhibits the expression of SDF-1 and a substance that inhibits the expression control site such as a promoter.

The antibodies described above that can be used in this invention, such as anti-SDF-1 antibodies and

anti-CXCR4 antibodies can be prepared as polyclonal or monoclonal antibodies using techniques that are known in the art. Especially, monoclonal antibodies derived from mammals are preferred as the antibodies that are used in the invention. The monoclonal antibodies derived from mammals include those produced by hybridomas and those produced by the hosts that have been transformed with expression vectors containing antibody genes by techniques in genetic engineering. These antibodies are those which possess the above-mentioned properties.

Antibody-producing hybridomas can principally be prepared using techniques known in the art in the following manner. That is, a desired antigen is used as the sensitizing antigen to carry out immunization according to a conventional immunization method; and the resulting immunized cells are fused with parent cells known in the art by a conventional cell fusion method, and monoclonal antibody-producing cells are subjected to cloning by a conventional cloning method.

The mammals to be immunized with the sensitizing antigen are not particularly limited, but should preferably be selected in consideration of their compatibility with the parent cells to be used in the cell fusion. Generally, animals belonging to the rodent family, such as mouse, rat and hamster are

used. Immunization of animals with the sensitizing antigen is carried out by a method known in the art.

For the mammalian myeloma cells used as the other parent cells for fusion with the immunocytes, a variety of cell lines already known in the art may appropriately be employed. The cell fusion between the immunocytes and myeloma cells may be carried out basically according to a conventional method, such as the method of Milstein et al (Kohler, G. and Milstein, C., Methods Enzymol., 73, 3-46 (1981)) with any possible modifications.

The obtained hybridomas are selected by culturing in a common selection medium, such as HAT medium (medium containing hypoxanthine, aminopterin and thymidine). Culturing in the HAT medium is continued for a sufficient time to allow killing of all the cells other than the targeted hybridomas (the non-fused cells), which is usually from a few days to a few weeks. The usual limiting dilution method is then performed for screening and cloning of hybridomas producing the target antibodies. Antibodies may be acquired from the thus-obtained hybridomas following a method that is commonly employed.

In addition to acquisition of the hybridomas by immunizing an animal other than human with the antigen as described above, human lymphocytes are sensitized

in vitro with a desired antigen protein or antigen-expressing cell, and the sensitized B lymphocytes are fused with human myeloma cells, such as U266, whereby desired humanized antibodies possessing the binding activity toward the desired antigen or antigen-expressing cell may be obtained. Furthermore, the desired humanized antibodies may be acquired according to the aforementioned method by administering the antigen or the antigen-expressing cell to a transgenic animal having a reparatory of human antibody genes.

An antigen gene is cloned as a monoclonal antibody from the hybridoma and incorporated into a suitable vector; and this is introduced into a host, and the gene manipulation technology is used to produce a recombinant antibody, which may then be used in this invention. See, for example, Carl, A. K. Borrenbaeck, James, W. Larrick, Therapeutic Monoclonal Antibodies, published in the United Kingdom by Macmillan Publishers Ltd. 1990.

In this invention, recombinant antibodies that have been artificially modified for the purpose of lowering heterogeneous antigenicity against humans, such as chimeric antibodies (European Patent Publication EP125023) and humanized antibodies (European Patent Publication EP125023), may be used. These antibodies can be produced by known methods.

The chimeric antibody comprises the variable region of an antibody derived from a mammal other than human and the constant region (C region) derived from a human antibody. The humanized antibody comprises a complementarity-determining region of an antibody derived from a mammal other than human, a framework region (FR) derived from a human antibody and a C region. They are useful as the effective ingredients in this invention because of diminished antigenicity in the human body.

The antibodies used in this invention may be fragments of antibody or modified substances thereof insofar as they can desirably be used in the invention. The fragments of antibody, for example, include Fab, $F(ab')_2$, Fv, and single chain Fv (scFv) obtainable by linking Fv from H chain and Fv from L chain via a suitable linker. Specifically, an antibody is treated with an enzyme such as papain or pepsin to produce antibody fragments. Alternatively, genes encoding these antibody fragemnets are constructed and are introduced into an expression vector, after which they are expressed in suitable host cells.

The phage library method can be utilized to obtain the antibodies that are used in this invention. (Marks, C. et al., The New England Journal Medicine 335, 730-733). For example, a cDNA library is

acquired from human B cells that comprises a human antibody V region, such as the gene encoding scFv. This cDNA library is introduced into a phage vector such as the M13 phage surface presenting vector, and this is allowed to infect *E. coli*. The cDNA library is expressed in *E. coli*, and the antibody V region is produced on the cell surfaces. If selection is made on a plate coated with the desired antigen based on antigen-binding activity, a gene encoding the desired antibody can be obtained.

Antibodies possessing stronger binding activity to antigens may be obtained by the chain shuffling method with application of the phage library method. (Akamatsu, Y. and Tsurushita, N. Medical Immunology 27, 273-286 (1994)). Specifically, one member of the V region of an antibody gene that has been separated (such as VH) is fixed; and a new library is constructed from the mixture of the one member and the other member prepared from B cells (such as VL). Clones that bind to the antigen more strongly than do the others may be separated from the library.

It is also possible to obtain antibodies possessing stronger binding activity to antigens by introducing artificial mutations into the amino acid sequences of the antibodies. (Akamatsu, Y. and Tsurushita, N. Medical Immunology 27, 273-286 (1994)).

More specifically, the mutation is introduced into a gene encoding the cloned antibody V region, and this gene is expressed by the phage library method described above. Thereby, it becomes possible to obtain a gene encoding the antibody that possesses stronger binding activity to antigen.

These fragments of antibody can be produced by hosts after their genes are acquired and expressed in the same manner as described previously above. As used in the present specification, "antibody" encompasses these fragemnts of antibody.

Modified forms of antibody can employ antibodies that are bound to various molecules such as polyethylene glycol (PEG). As used in the present specification, the "antibody" encompasses these modified forms of antibody. Acquisition of these modified forms of antibody can be done by subjecting the obtained antibodies to chemical modification. These methods have already been established in the art.

The antibodies that are expressed and produced as described above can be separated from the host cells intracellularly or extracellularly, and can be purified to homogeneity according to methods that are commonly used. Concentration measurement can be carried out by the measurement of absorbance, ELISA or the like.

For the CXCR4 inhibitor used in this invention, there is mentioned a protein having an SDF-1- or CXCR4-like structure (structure-resembling protein). This substance is one that possesses binding activity to SDF-1 or CXCR4 and that does not transmit its biological activity. That is, it blocks signaling by SDF-1, because it binds to CXCR4 in a competitive manner with SDF-1, but does not transmit the biological activity of SDF-1.

SDF-1 structure-resembling proteins may be prepared by introducing mutations into the amino acid sequence of SDF-1 through substitution of amino acid residues thereof. For SDF-1 on which the SDF-1 structure-resembling proteins are based, its source does not matter; however, it is preferably human SDF-1 in consideration of its antigenicity or the like. Specifically, the amino acid sequence of SDF-1 is used to predict its secondary structure by using a molecular modeling program known in the art such as WHATIF (Vriend et al., J. Mol. Graphics 8, 52-56 (1990)); and further the influence on the whole, of the amino acid residue to be substituted is evaluated to carry out the preparation.

After a suitable amino acid residue to be substituted has been determined, a vector containing the base sequence that encodes human SDF-1 gene is

used as a template, and the introduction of mutation is carried out by the PCR method (polymerase chain reaction), which is commonly done, so that the amino acid may be substituted. This allows a gene encoding the SDF-1 structure-resembling protein to be obtained. This gene is incorporated into a suitable expression vector as appropriate, and the SDF-1 structure-resembling protein can be obtained according to the methods for the expression, production and purification of the recombinant antibodies as described previously. SDF-1 of which the N-terminus has been deleted is known as an SDF-1 structure-resembling protein (EMBO J. 16, 6996-7007 (1997)).

The SDF-1 partial peptide or the CXCR4 partial peptide that is used in this invention is a substance that possesses binding activity to CXCR4 or to SDF-1 and that does not transmit the biological activity of SDF-1. That is, the SDF-1 partial peptide or the CXCR4 partial peptide specifically inhibits SDF-1 from binding to CXCR4, because each binds to CXCR4 or to SDF-1 and traps either of them.

Consequently, they block signaling by SDF-1, because they do not transmit the biological activity of SDF-1.

The SDF-1 partial peptide or the CXCR4 partial peptide is a peptide comprising a part or the whole of

the amino acid sequence of the region, which is responsible for the binding between SDF-1 and CXCR4, in the amino acid sequence of SDF-1 or of CXCR4. Such a peptide usually comprises 10-80 amino acid residues, preferably 20-50 amino acid residues, and more preferably 20-40 amino acid residues.

The SDF-1 partial peptide or the CXCR4 partial peptide can be prepared by identifying in the amino acid sequence of SDF-1 or of CXCR4, the region that is responsible for the binding between SDF-1 and CXCR4 and by preparing a part or the whole of the amino acid sequence of said region according to a method that is commonly known, such as a technique in genetic engineering or peptide synthesis.

To prepare the SDF-1 partial peptide or the CXCR4 partial peptide by the technique in genetic engineering, a DNA sequence encoding the desired peptide is incorporated into an expression vector, and the methods for expression, production and purification of the recombinant antibodies as described previously are followed with any possible modifications, thus enabling the preparation.

To prepare the SDF-1 partial peptide or the CXCR4 partial peptide by peptide synthesis, methods that are commonly used in the peptide synthesis, such as the solid phase synthesis or the liquid phase

synthesis, can be employed. Concretely, the method as described in "Development of Drugs, Peptide Synthesis Vol. 14, Ed. by Nariaki Yajima, Hirokawa Publisher (1991) may be followed with any possible modifications. For the solid phase synthesis, the following method is, for example, employed: an amino acid corresponding to the C-terminus of the peptide to be synthesized is allowed to bind to a support insoluble in an organic solvent; and the reaction wherein an amino acid protected with suitable protecting groups at its α-amino group and side-chain functional group is condensed to the foregoing amino acid one amino acid at a time in the direction of from the C-terminus to the N-terminus and the reaction for detaching the protecting group for the α-amino group of the amino acid or peptide bound to the resin are alternately repeated to elongate the peptide chain. The solid phase peptide synthesis is largely classified into the Boc method and the Fmoc method, depending on the kind of protecting groups to be used.

After the objective peptide is synthesized in this manner, deprotection reaction is done and the peptide is cleaved from the support for the peptide chain. In the cleavage reaction from the peptide chain, the Boc method can usually employ hydrogen fluoride or trifluoromethanesulfonic acid and the Fmoc

method can usually employ TFA. In the Boc method, the protected peptide resin mentioned above is, for example, treated in hydrogen fluoride in the presence of anisole. Subsequently, deprotection of the protecting group and cleavage from the support recovers the peptide.

The product is lyophilized to yield a crude peptide. In the Fmoc method, deprotection reaction and cleavage reaction from the support for the peptide chain can also be carried out in TFA using manipulations similar to those mentioned above.

The resulting crude peptide can be separated and purified by being applied on HPLC. The elution may then be carried out under optimum conditions with a solvent of the water-acetonitrile system that is usually used to purify proteins. The fractions corresponding to the peaks of the obtained profile of chromatography are fractionally separated and lyophilized. The peptide fractions thus purified are identified by molecular weight analysis through mass spectrometry, amino acid composition analysis, amino acid sequencing, or the like.

For the SDF-1 partial peptide or the CXCR4 partial peptide that is used in this invention, its sequence does not matter insofar as each binds to CXCR4 or to SDF-1 and possess no signaling activity.

The amino acid sequences that are already known can be used both for the SDF-1 partial peptide and the CXCR4 partial peptide. For example, when the ligand is SDF-1, the amino acid sequences set forth in SEQ ID NO: 5 (human) and SEQ ID NO: 7 (mouse) are usable.

There is no particular limitation to the substance that potentiates the action due to CXCR4 that can be used in this invention. For the substance that potentiates SDF-1, there are mentioned SDF-1 itself, an agonist of SDF-1, and a potentiator of SDF-1 expression. Furthermore, for the substance that potentiates a CXCR4 receptor, there are mentioned CXCR4 itself, an agonist of CXCR4, and a potentiator of CXCR4 expression.

As explained above, use of the vascularization inhibitor according to this invention comprising the CXCR4 inhibitor as the effective ingredient allows for the inhibition of vascularization; therefore, it will exert an antitumor effect (inhibition of neovascularization) on solid cancer in addition to antitumor effects on angiosarcoma (cancer of blood vessels themselves) and Kaposi's sarcoma. It will also exert therapeutic effects against diseases pathologically caused by neovascularization, such as chronic articular rheumatism, psoriasis, and diabetic retinopathy.

If the therapeutic agent for a disease pathologically caused by neovascularization which comprises the substance that potentiates the action of CXCR4 is used, it will be possible to promote neovascularization. The use will exert therapeutic effects on myocardial infarction and diseases involving neovascularization after surgery, such as wound healing, repairing and remodeling of bones, repairing of cartilage, growth of hair, myocardial infarction, brain infarction, and brain trauma.

For the neovascularization inhibition and promotion test methods that can be used in this invention, a neovascularization assay may be employed. There is no particular limitation to this assay, and a method that is commonly known can preferably be used. ("Research Manual for the Invasion and Metastasis of Cancers" the Cancer Metastasis Study Group Ed., Kinhodo, 159-182, (1994)). Specifically, among others, there are mentioned (I) the method for measuring cleavage of the spaces between vascular endothelial cells (i.e., effect on the vascular endothelial cells) which is based on the finding that the vascular endothelial cells rupture when tumor cells invade out of the blood vessels (from the permeability of FITC-dextran); (II) the cornea method known as an *in vivo* measurement method for identifying a candidate factor

that exerts the function of a neovascularization-inducing factor *in vivo*; (IV) CAM method (chickembryochorioallantoic membrane); (V) the dorsal subcutanea method for measuring the quantity of induced blood vessel by the naked eyes; and (VI) the method for determining the formation of lumen by vascular endothelial cells.

To confirm the antitumor effect, there may be mentioned *in vivo* experiments using a transplant model or transplant metastasis model and *in vitro* experiments with cancer cells. Specifically, the method described in "Research Manual for the Invasion and Metastasis of Cancers" the Cancer Metastasis Study Group Ed., Kinhodo, 7-158 (1994) may be used.

The vascularization inhibitor, anti-sold cancer agent, therapeutic agent, tissue-repairing agent, or the like according to this invention may be systemically (by oral route) or locally administered. For example, intravenous injection (such as intravenous drip infusion), intramuscular injection, intraperitoneal injection, or subcutaneous injection may be selected: an appropriate method of administration may be selected depending on the age or the severity of the subject.

The effective unit dose is chosen within the range of from 0.09 mg to 100 mg per kg of body weight.

Alternatively, a dose of 1-1000 mg, preferably a dose of 5-50 mg for the subject may be chosen.

The vascularization inhibitor, anti-sold cancer agent, therapeutic agent, tissue-repairing agent, or the like according to this invention may together contain pharmaceutically acceptable carriers or additives, depending on the route of administration. Examples of such carriers and additives include water, organic solvents that are pharmaceutically acceptable, collagen, poly(vinyl alcohol), poly(vinylpyrrolidone), carboxyvinylpolymer, sodium carboxymethyl cellulose, sodium polyacrylate, sodium arginate, water-soluble dextran, sodium carboxymethyl starch, pectin, methylcellulose, xanthan gum, gum arabic, casein, gelatin, agar, diglycerin, propylene glycol, poly(ethylene glycol), vaseline, paraffin, stearyl alcohol, stearic acid, human serum albumin (HSA), mannitol, sorbitol, lactose, surfactants that are accepted as drug additives, etc.

The additives to be used may appropriately (or in combination) be selected among those mentioned above depending on the dosage form, but are not limited thereto.

This invention will be hereinbelow illustrated in greater details by way of examples; however, the invention is in no way limited by those examples.

EXAMPLES

Creation and analysis of mice completely lacking CXCR4

Genome DNA containing the CXCR4 locus was isolated from a murine cell line 129DNA library (STRATAGENE).

A 1.1-kb genomic fragment containing the 5'-coding region of exon 2 was replaced by the neomycin resistant gene, and the herpes simplex thymidine kinase gene was linked to the 5'-terminus.

The targeting vector was introduced into the cells on day 14.1 of embryogenesis (referred to as "E14.1" hereafter) by electroporation, and a homologous recombination was selected by the use of G418 and ganciclovir and identified by PCR.

The structure of the mutant locus and the presence of a single insert in the ES cell colony were confirmed by Southern hybridization. According to the method as described in Nagasawa, T. et al., Nature 382, 685-688 (1996), the mutant ES cell colony was used to create a mutant mouse by the injection of undifferentiated embryonic cells.

For Southern hybridization, tail DNA was digested with EcoRI, transferred to a nylon membrane, and hybridized to 550-bp probe A with the 5'-homology region.

RT-PCR was performed for 40 cycles, using 3 μg of total RNA isolated from the fetal liver of E18.5 embryos as a starting material according to the standard method. A 630-bp PCR product was amplified using CXCR4-specific primers, i.e., forward primer (SEQ ID NO: 11) and reverse primer (SEQ ID NO: 12). Histological analysis and flow cytometry analysis were carried out substantially according to the method as described in Nagasawa, T. et al., Nature 382, 685-688 (1996).

Immunohistostaining substantially followed the method of Adachi, S., Yoshida, H., Kataoka, H. Nishikawa, S.-I. Int. Immunol. 9, 507-514. The sections of embryos and organs were fixed with 4% paraformaldehyde, dehydrated with methanol, decolorized with 30% hydrogen peroxide in methanol, and hydrated again.

After incubating in PBSMT (PBS containing 1% skim milk powder and 0.3% v/v TritonX-100), the sample was incubated with diluted anti-PECAM antibody (1:250)(PharMingen) in PBSMT at 4 °C overnight. Then, the sample was washed with PBSMT, and incubated with diluted horseradish peroxidase labeled anti-rat Ig antibody (1:500)(Biosource) in PBST at 4 °C overnight.

Subsequently, the sample was thoroughly washed. The embryo was incubated in PBS containing 250 μg/ml

diaminobenzidine (Dojin Chemicals) and 0.08% $NiCl_2$ for 30 min. Hydrogen peroxide was added to the sample to provide a final concentration of 0.01%, after which peroxidase staining was carried out. The reaction was quenched after about 30 min.

According to the method as described in Nagasawa, T. et al., Nature 382, 685-688 (1996), a fragment of murine CXCR4 or PBSF/SDF-1 cDNA was used as a probe to carry out antisense transcription.

Mice lacking CXCR4 were created to determine the physiological function of CXCR4. Specifically, a targeting vector was constructed, such that most of exon 2 within the CXCR4 gene which contained all transmembrane-spanning regions critical to the receptor function had been deleted and the deleted portion would be replaced by the neomycin resistant gene (neo). This would result in that after homologous recombination, the complete CXCR4 gene would be substantially deleted.

Fig. 1 is a graph showing a targeting strategy for the CXCR4 gene (denoted as top, middle, and bottom). There are shown the CXCR4 wild-type allele at the top, a targeting vector in the middle, and a predicted mutant allele at the bottom. The coding regions of the genes are indicated by black boxes. Empty boxes indicate the 5'- and 3'-untranslated

regions. Dotted lines indicate homologous fragments used in the targeting vector. Probe A is an external probe for Southern hybridization. Here, restriction sites are E (EcoRI), Sh (SphI), and X (XhoI), respectively.

Fig. 2A is a photograph showing the Southern blot analysis of tail DNAs from wild-type (+/+) and heterozygous mutant (+/-) mice. The EcoRI-EcoRI fragments from the 11.8-kb wild-type and the 8.2-kb targeted allele which were identified by probe A are shown in the figure. Fig. 2B is a photograph showing the RT-PCR amplification analysis of CXCR4 expression. Total RNAs were prepared from E18.5 wild-type and homozygous mutant embryos, and amplified with CXCR4-specific primers. The RT-PCR amplification employed G3PDH mRNA, which was universally expressed, as a control for the presence of any amplifiable RNA.

Mice with a $CXCR4^{+/-}$ heterozygous mutation were created. The mice were healthy and fertile. $CXCR4^{-/-}$ homozygous mutant embryos were present at the expected ratios until E15.5 of embryogenesis. However, about half of the $CXCR4^{-/-}$ embryos were dead at E18.5 and $CXCR4^{-/-}$ neonates died within an hour similarly to mice lacking PBSF/SDF-1 as previously reported (Nagasawa, T. et al., Nature 382, 685-688 (1996)).

To elucidate the functions of CXCR4 during embryogenesis, expression of CXCR4 in developing embryos was examined by *in situ* hybridization. High levels of CXCR4 transcripts were detected in the endothelium of developing blood vessels during embryogenesis.

Based on this finding, the effect of CXCR4 gene deficiency on vascularization was investigated. On visual inspection, vitellin and umbilical vessels were normal. Histological examination of E18.5 $CXCR4^{-/-}$ embryos demonstrated the presence of the major blood vessels, including aorta, vena cava, carotid artery, jugular vein, coeliac artery and superior mesenteric artery and superior mesenteric vein. To visualize the vascular system of organs, whole-mount preparations of wild-type and mutant embryos were then immunostained with an anti-PECAM-1 antibody. It is known that PECAM-1 is specifically and stably expressed in all endothelial cells during embryonic period. (Vecchi, A. et al. Eur. J. cell Biol. 63, 247-255 (1994); Baldwin, H. S. et al., Development 120, 2539-2558 (1994)).

Consequently, in the gastrointestinal tract, including stomach, intestine and mesentery, a highly branched homogeneous vascular network was observed in both the wild-type and the mutant by E11.5. The formation of large and small vessels through

remodeling in the mesentery connecting to mid-gut loop was observed at around E12.5. As Fig. 3 shows, many large branches of superior mesenteric artery and vein supplying nutrient to the intestine were formed in wild-type embryos at E13.5. On the other hand, these large branches were not present in the mesenteries of $CXCR4^{-/-}$ embryos at E13.5; instead, only small vessels were formed. Histological analysis by microscopy revealed superior mesenteric arteries and veins in the mesenteries of wild-type embryos at E13.5. This showed that the branched vessels were paired between the artery and the vein (Fig. 4). In contrast, most of the vessels in $CXCR4^{-/-}$ embryos were not paired, but were single, as can be seen in Fig. 8. However, the superior mesenteric arteries and veins within the mesenteries of the wild-type embryos were normal. In E17.5 wild-type embryos, the large mesenteric vessels split into many branches and reach the intestine (Figs. 5 and 6). However, in the $CXCR4^{-/-}$ embryos such vessels corresponding to the large mesenteric vessels were substantially absent (Figs. 6 and 9). Also, a few large vessels with aberrant branching were observed in the mutant mesenteries (Figs. 6 and 9). In most of E16.5 mutant embryos multiple haemorrhagic lesions were observed in their small intestines because of such defective vascular system. This

pathogenesis is believed to be the result of aberration of the circulatory system governing the intestine (Fig. 11).

The above-mentioned results have demonstrated that CXCR4 is essential for the normal vascularization of the small intestine: the mechanism is believed to be due to that CXCR4 is involved in the branching and/or remodeling of the mesenteric vessels.

In the stomach, large vessels branching out from mesenchymal vessels along the lesser curvature were formed and distributed to the entire ventral and dorsal surfaces in the wild-type embryos by E13.5 (Figs. 12A and 12C). Histological analysis revealed that these vessels were paired between the artery and the vein in the E15.5 wild-type mice as the inset in Fig. 12 C shows. However, the corresponding vessels were not found in the mutant embryos (Figs. 12B and 12D). Formation of the network of small vessels that surrounds the stomach seemed to be normal in the mutant embryos (Fig. 12D).

Histological analysis of the stomachs and intestines of E18.5 mutant embryos detected no obvious abnormalities in organogenesis. For example, the smooth muscle layers (both the outer and inner layers) of the gastrointestinal tract of mutant mice seemed to be normal in the longitudinal and vertical directions.

Figs. 3-6 are photographs showing defects of the gastrointestinal vessels in the $CXCR4^{-/-}$ embryos, and also photographs showing immunohistostaining of mesenteries and intestines of the wild-types with the anti-PECAM antibody. Fig. 3 shows the mesentry and mid-gut loop regions at E13.5. Fig. 5 shows a jejunum at E17.5. Fig. 6 shows a jejunum at E17.5. Fig. 7 shows a cross-section of the stained mesentry at E13.5. The arrows in Figs. 3, 5, and 6 indicate large branches of superior mesenteric artery or superior mesenteric vein supplying nutrient to the small intestine, in wild-type mesentries.

Similarly, Figs. 7-11 are photographs showing defects of the gastrointestinal vessels in the $CXCR4^{-/-}$ embryos, and also photographs showing immunohistostaining of mesenteries and intestines of the mutants with the anti-PECAM antibody. Fig. 7 shows the mesentry and mid-gut loop regions at E13.5. Fig. 9 shows a jejunum at E17.5. Fig. 10 shows a jejunum at E17.5. Fig. 7 shows a cross-section of the stained mesentry at E13.5. Fig. 11 shows the haemorrhagic lesions of unstained intestine of a mutant mouse at E16.5. The arrows in Figs. 9 and 10 indicate large vessels displaying aberrant running and/or branching in the mutant mice.

Figs. 12A-12D are photographs showing the results of immunohistostaining stomachs with the anti-PECAM antibody. Figs. 12A and 12B show E13.5; Figs. 12C and 12D show E15.5; Figs. 12A and 12C show the wild types; and Figs. 12B and 12D show the mutants. The inset in the photograph of Fig. 12C shows a haematoxylin-and-eosin-stained section of large vessels in the wall of stained stomach at E15.5. The arrows in Figs. 12A and 12C indicate large vessels only observed in the mutant. "du" represents duodenum; "p" represents the proximal part of mid-gut loop; "dm" represents the distal part of mid-gut loop; "a" represents artery; and "v" represents vein.

These findings indicate that the abnormalities in vascularization in the $CXCR4^{-/-}$ mice are not a secondary outcome of those in the gastrointestinal tracts themselves. Abnormalities similar to those in vascularization were also observed in the mice lacking PBSF/SDF-1.

In situ hybridization analysis indicated that CXCR4 transcripts were expressed in the endothelial cells of blood vessels in the mesentery and in the wall of the intestine and stomach in the E12.5 wild-type embryos (Figs. 13B and 13E). Particularly, strong expression was observed in the endothelial cells of branches arising from the superior mesenteric

arteries (Figs. 13B and 13E). In contrast, PBSF/SDF-1 was expressed at high levels in mesenchymal cells surrounding the endothelial cells of the mesentry, but not in the endothelial cells or the wall of intestine and stomach (Fig. 13C).

Figs. 13A-13F are photographs showing an analysis of CXCR4 and PBSF/SDF-1 expression in the gastrointestinal tract through *in situ* hybridization. Serial sections of the wild-type mesentery connecting to the mid-gut loop were used; one piece was stained with haematoxylin and eosin (Figs. 13A and 13D); another piece was hybridized to the CXCR4-specific probe (Figs. 13B and 13E); an additional piece was hybridized to the PBSF/SDF-1-specific probe (Fig. 13E). Figs. 13D and 13E are enlargements of branched vessels arising from the superior mesenteric artery shown in Figs. 13A and 13B, indicating strong expression of CXCR4 in endothelial cells of the vessel. The arrows and arrowheads in Figs. 13B and 13E indicate the endothelial cells of mesenteric blood vessels with observed CXCR4 expression. PBSF/SDF-1 is expressed in the mesenchymal cells surrounding endothelial cells in the mesentery (Fig. 13E). Fig. 13F is a cross-section of bone marrow of the E18.5 wild-type embryo, showing CXCR4 expression in haemotopoietic cells but not in spindle-shaped stromal cells. "m" represents

mesentry; "i" represents intestine; "a" represents superior mesenteric artery; and "v" represents superior mesenteric vein.

The expression patterns obtained indicate that PBSF/SDF-1 produced by mesenchymal cells acts on CXCR4 on endothelial cells; and this strongly suggests the presence of the paracrine signal by a cytokine that plays a extremely important role in the mesenteric mesenchyme. Thus, the phenotype that lacks large vessels in the stomachs of $CXCR4^{-/-}$ and $PBSF/SDF-1^{-/-}$ may result from abnormalities in the vascular branching and/or remodelling in the mesenchyme along the lesser curvature of the stomach.

To examine the vascular systems of other organs, a whole-mount yolk sac, brain and heart were stained with an anti-PECAM-1 monoclonal antibody. There was no obvious difference between $CXCR4^{-/-}$ and $PBSF/SDF-1^{-/-}$, and the wild-type in the formation of large and small vessels in the whole-mount yolk sac (E12.5, E14.5), the head region (E11.5) and the heart (E12.5-E14.5).

In summary, the aforementioned experimental results have shown that CXCR4 and PBSF/SDF-1 are essential for the formation of a mature vascular system which is supplied to the gastrointestinal tract by acting on the endothelial cells of blood vessels and regulating vascular branching and/or remodelling.

A flow cytometric analysis revealed that the number of B-cell progenitors in fetal livers of CXCR4$^{-/-}$ mice was severely reduced. Histological analysis did not detect myelocytes and their progenitors in the medullary cavity. In addition, defects of the membranous portion of the cardiac ventricular septum were found in the hearts of E18.5 mutant mice. These abnormalities are very similar to the phenotype found in the mice lacking PBSF/SDF-1, supporting the thinking that CXCR4 is a primary physiological receptor for PBSF/SDF-1.

In the E18.5 wild-type embryonic bone marrow as determined by *in situ* hybridization, CXCR4 transcripts were expressed in hematopoietic cells, while they were not expressed in spindle-shaped stroma cells where the expression of PBSF/SDF-1 transcripts had been observed (Fig. 3D). The results of these expression patterns mean the presence of paracrine signaling in bone marrow.

Analyses of receptor tyrosine kinases (RTKs), such as Flk-1 and Tie-2, and their ligands, such as VEGF, angiopoietin-1 and PDGF-B that have hitherto been reported, using mutant mice have indicated that they play an important role in development of the vascular system, and that many of them are required for the very early stage of vascularization in genesis

as well as for vascularization in all parts of body, including the york sac and the extra-embryonic vasculature. (Shalaby, F. et al., Nature 376, 62-66 (1995); Fong, G. -H., Rossant, J., Gertsenstein, M. & Breitman, M. L., Nature 376, 66-70 (1995); Dumount, D. H. et al., Genes Dev 8, 1897-1909 (1994); Sato, T. N. et al., Nature, 376, 70-74 (1995); Carmeliet, P. et al., Nature, 880, 435-439 (1996); Ferrara, N. et al., Nature, 380, 439-442 (1996); and Suri, C. et al., Cell 87, 1171-1180 (1996)).

In contrast, the functions of CXCR4 and PBSF/SDF-1 operate at later stages of genesis and are organ-specific. Tie-2 and its ligand, angiopoietin-1, are thought to be necessary for the branching and/or remodelling in the early vascular system. (Sato, T. N. et al., Nature 376, 70-74 (1995); Suri, C. et al., Cell 87, 1171-1180 (1996)). Their roles in the formation of the mature vascular system in the gastrointestinal tract are not clear. Obvious abnormalities found in the York sac vascular system of Tie-2 or angiopoietin-$1^{-/-}$ mice were not noted in the CXCR4 or PBSF/SDF-$1^{-/-}$ mice. CXC chemokines, such as PF4 (Maione, T. E. et al., Science 247, 77-79 (1990)), IL-8 (Koch, A. E. et al., Science 258, 1798-1801 (1992)), IP-10 (Luster, A. D. et al., J. Exp. Med. 182, 219-231 (1995)), and Groβ (Cao, Y. H., et al., J. J.

Exp. Med. 182, 2069-2077 (1995)), have been reported to be neovascularization regulators. However, expression of their receptors in endothelial cells and their physiological roles have not yet been elucidated. Although coagulation factor V (Cui, J., et al., Nature, 384, 66-68 (1996)) and tissue factor (Carmeliet, P. et al., Nature 883, 75-78 (1996)) have been shown to be essential for the york sac vascular system, it is not clear as to what kind of acceptor mediates these factors.

With the background mentioned above, it can be said that this invention have demonstrated the presence of a novel signaling system--chemokines and a seven-transmembrane spanning, G-protein-coupled receptors--essential for vascularization.

It has recently been shown that mice lacking the α-subunit of the heterotrimeric GTP-binding protein Gα13 have abnormalities, such as no formation of a york-sac vascular system and enlargement of embryonic small vessels. Although the phenotypes are different from those of mice lacking CXCR4, it is necessary to examine the possibility of CXCR4's coupling to Gα13.

It is known that CXCR4 and CCR5 are essential co-receptors when T-cell line tropic and macrophage-tropic HIV-1 strains infect host cells, respectively.

(Feng, Y., et al., Science 272, 872-877 (1996); Fauci, A. S., Nature 884, 529-584 (1996)). Between the two, the people homozygous for CCR5 deletion have been discovered; and they are resistant to HIV-1 infection and have no obvious health problems. (Liu, R et al., Cell 86, 367-377 (1996); Samson, M. et al., Nature 382, 722-725 (1996); and Dean, M. et al., Science 273, 1856-1861).

Concerning the other CXCR4, it has, however, been strongly suggested that homologous CXCR4 deletion is unlikely to occur in humans since mice lacking CXCR4 are apt to die *in utero*. There still remains the possibility that additional genetic factors or homologous viable mutations may exist in long-term survivors who are resistant to T cell-line-tropic HIV-1.

Industrial Applicability

Based on the finding of this invention that vascularization is suppressed in CXCR4 knockout mice, a vascularization inhibitor comprising as the effective ingredient, a substance that inhibits the action of CXCR4 can be prepared: since vascularization is essential for the maintenance and enlargement of cancerous tissues, the finding is utilized in the preparation of an anti-solid cancer agent and a therapeutic agent for a disease pathologically caused

by neovascularization, which comprises as the effective ingredient a substance that inhibits the action of CXCR4, as well as in the preparation of a tissue-repairing agent comprising as the effective ingredient, a substance that potentiates the action of CXCR4.

CLAIMS

1. A therapeutic agent for inhibiting vascularization comprising as the effective ingredient, a substance that inhibits the action due to CXCR4.

2. A therapeutic agent for a solid cancer comprising as the effective ingredient, a substance that inhibits the action due to CXCR4.

3. A therapeutic agent for a disease pathologically caused by neovascularization comprising as the effective ingredient, a substance that inhibits the action due to CXCR4.

4. A therapeutic agent for repairing a tissue comprising as the effective ingredient, a substance that inhibits the action due to CXCR4.

5. The therapeutic agent according to any of claims 1-4, wherein the substance inhibits the very binding between SDF-1 and CXCR4.

6. The therapeutic agent according to any of claims 1-4, wherein the substance inhibits signaling from CXCR4 to nuclei.

7. The therapeutic agent according to any of claims 1-4, wherein the substance inhibits the very expression of CXCR4.

8. The therapeutic agent according to any of claims 1-4, wherein the substance inhibits the very expression of SDF-1.

9. The therapeutic agent according to claim 5, wherein the substance inhibits SDF-1.

10. The therapeutic agent according to claim 5, wherein the substance inhibits CXCR4.

11. The therapeutic agent according to claim 9, wherein the substance inhibits CXCR4 in antagonistic competition with SDF-1.

12. The therapeutic agent according to claim 9, wherein the substance inhibits SDF-1 from binding to CXCR4 by binding to SDF-1.

13. The therapeutic agent according to claim 11, wherein the substance is one selected from the group consisting of a SDF-1-like protein, a fused protein of the foregoing protein with another peptide or polypeptide, a partial peptide of SDF-1, and a low molecular weight compound having a structure similar to a binding site of SDF-1.

14. The therapeutic agent according to claim 12, wherein the substance is one selected from the group consisting of an anti-SDF-1 antibody, a fragment of said antibody possessing the activity of the anti-SDF-1 antibody, a fused protein possessing binding activity to SDF-1, a substance that induces a structural change in SDF-1, and a low molecular weight compound capable of binding to the CXCR4-binding site of SDF-1.

15. The therapeutic agent according to claim 10, wherein the substance inhibits CXCR4 in antagonistic competition with CXCR4 for binding to SDF-1.

16. The therapeutic agent according to claim 10, wherein the substance inhibits SDF-1 from binding to CXCR4 by binding to CXCR4.

17. The therapeutic agent according to claim 15, wherein the substance is one selected from the group consisting of a soluble CXCR4 that antagonizes CXCR4 in the inhibition, a protein having a CXCR4-like structure, a fused protein of the foregoing protein with another peptide or polypeptide, a partial peptide of CXCR4, and a low molecular weight compound having a structure similar to a binding site of SDF-1.

18. The therapeutic agent according to claim 16, wherein the substance is one selected from the group consisting of an anti-CXCR4 antibody, a fragment of said antibody possessing the activity of anti-CXCR4 antibody, a fused protein possessing binding activity to CXCR4, a substance that induces a structural change in SDF-1, and a low molecular weight compound capable of binding to the SDF-1-binding site of CXCR4.

19. The therapeutic agent according to claim 6, wherein the substance is an inhibitor of a signaling system located downstream of a G protein-coupled protein and is one selected from the group consisting

of a MAPK cascade inhibitor, a phosphoraipase C (PLC) inhibitor, and a PI3 kinase inhibitor.

20. The therapeutic agent according to claim 7, wherein the substance is a substance that causes apparent disappearance of CXCR4 from cells by acting on cell membrane to vary fluidity thereof and to cause disappearance of CXCR4 from the cell membrane.

21. The therapeutic agent according to claim 7, wherein the substance is a substance that inhibits the very expression of CXCR4 and is one selected from the group consisting of an antigene, an antisense polynucleotide, an antisense RNA expressed by an antisense vector, a ribozyme, and an inhibitor against the expression control site of CXCR4.

22. The therapeutic agent according to claim 8, wherein the substance is an antisense for the inhibition of expression of SDF-1.

23. The therapeutic agent according to claim 8, wherein the substance shows inhibition against the expression control site of SDF-1.

24. A method for suppressing vascularization comprising using a substance that inhibits the action due to CXCR4.

25. A method for treating a solid cancer comprising using a substance that inhibits the action due to CXCR4.

26. A method for treating a disease pathologically caused by neovascularization comprising using a substance that inhibits the action due to CXCR4.

27. A method for repairing a tissue comprising using a substance that inhibits the action due to CXCR4.

ABSTRACT

This invention provides a therapeutic agent for inhibiting neovascularization, a therapeutic agent for a solid cancer, a therapeutic agent for a disease pathologically caused by neovascularization, and a therapeutic agent for repairing a tissue comprising as the effective ingredient, a substance that potentiates the action of CXCR4.

Based on the finding that vascularization is suppressed in CXCR4 knockout mice, it becomes possible to prepare a therapeutic agent for suppressing vascularization, a therapeutic agent for a solid cancer, a therapeutic agent for a disease pathologically caused by neovascularization, each of which comprises as the effective ingredient, a substance that inhibits the action of CXCR4, as well as to prepare a therapeutic agent for repairing a tissue comprising as the effective ingredient, a substance that potentiates the action of CXCR4. Methods for treatment are made possible that use these therapeutic agents.

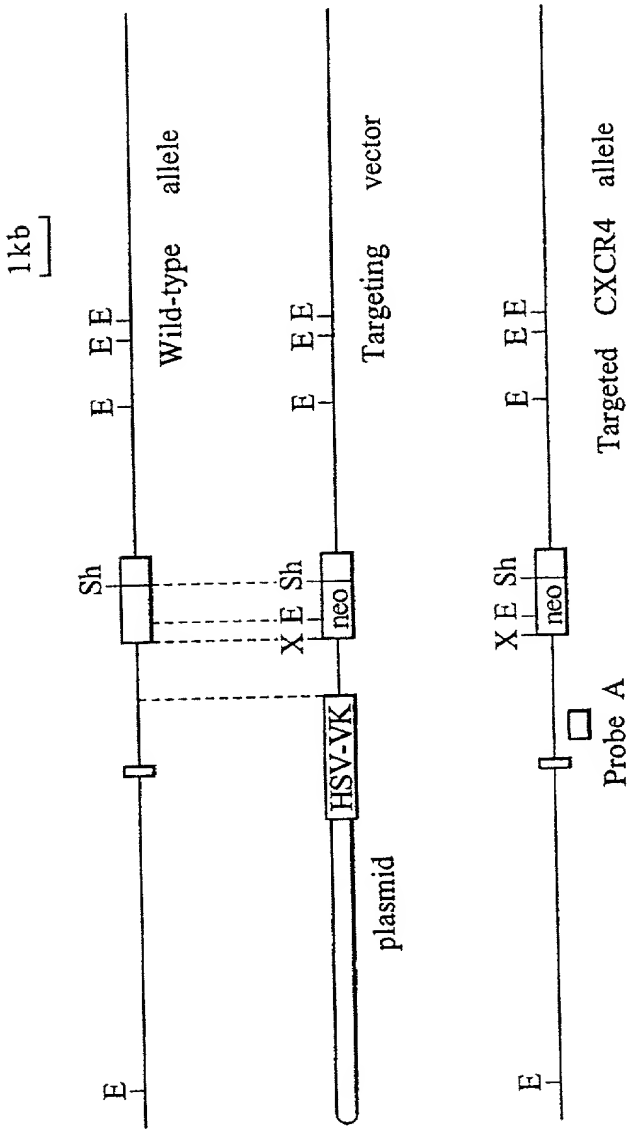


Fig.1

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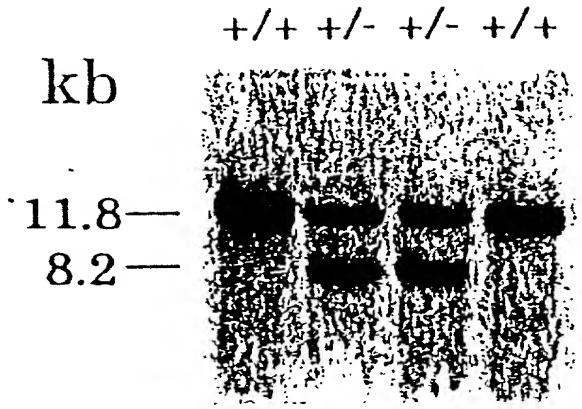

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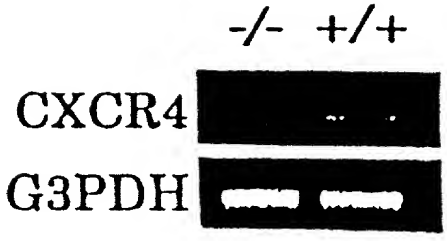

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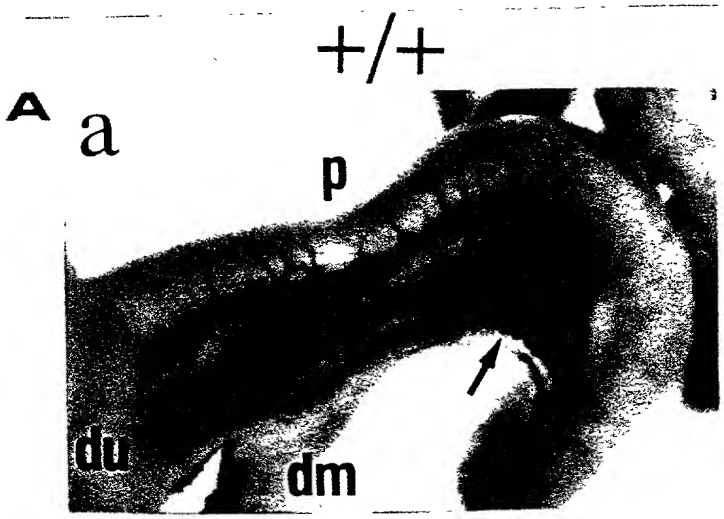

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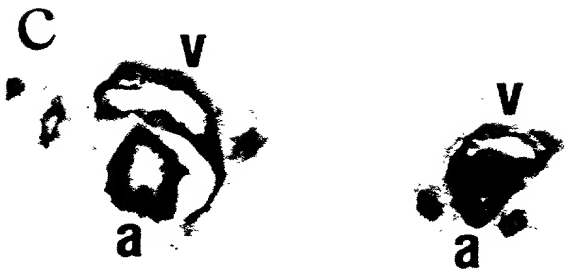

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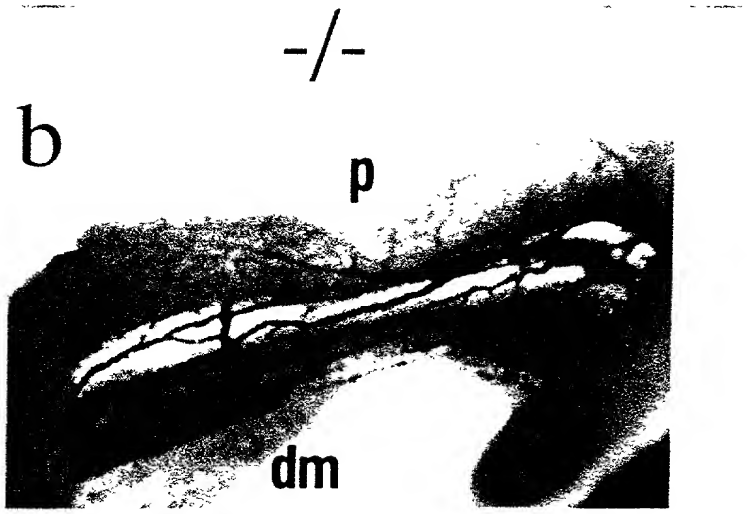

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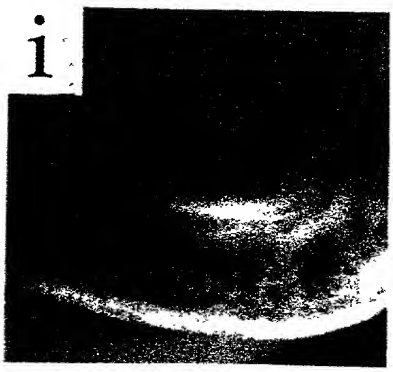

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図12B

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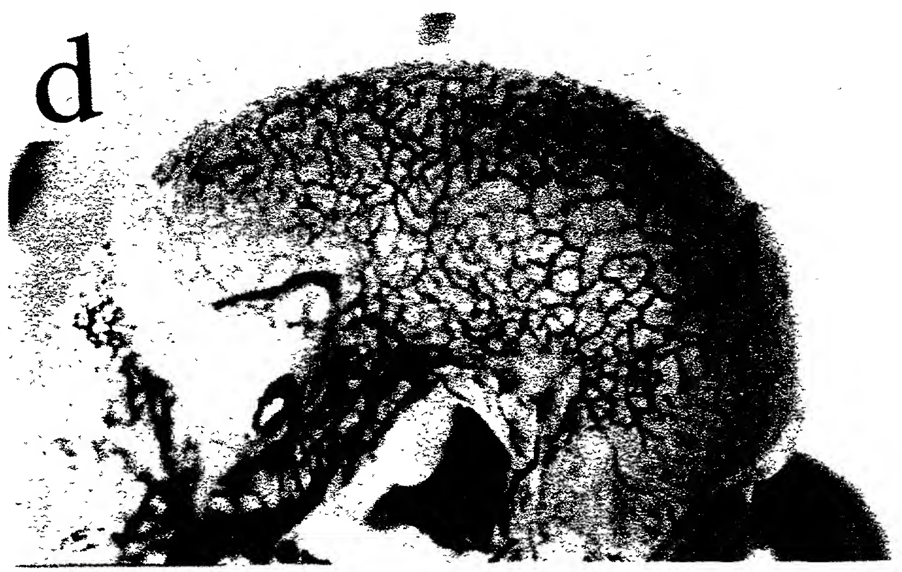

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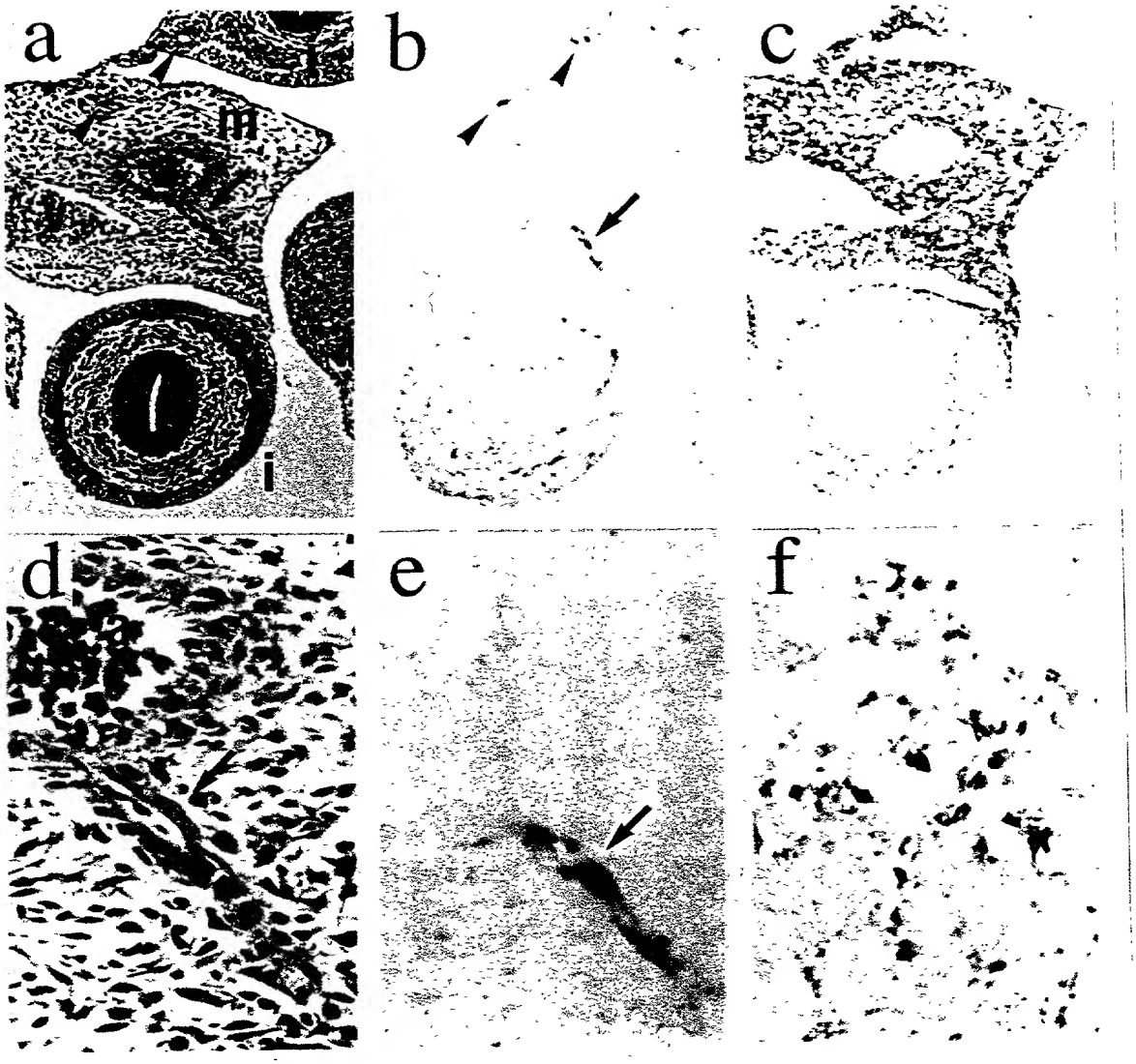

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MORGAN

Attorney's Docket No. _____

Combined Declaration and Power of Attorney

As a below named inventor, I hereby declare that:

This declaration is of the following type:

[ ] original [ ] supplemental

[X] national stage of PCT

[ ] divisional [ ] continuation [ ] continuation-in-part

My residence, post office address and citizenship are as stated next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

VASCULARIZATION INHIBITORS

the specification of which

[ ] is attached hereto.

[ ] was filed on _____
as United States Application Serial Number _____ and,
was amended on _____ (if applicable).

[X] was filed on March 23, 1999
as PCT International Application Number PCT/JP99/01448 and,
was amended under PCT Article 19 on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority under Title 35, United States Code, Section 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or PCT International application having a filing date before that of the application on which priority is claimed.

PRIOR FOREIGN APPLICATIONS, BENEFIT CLAIMED UNDER 35 USC §119(a)

Application Number	Country	Date of Filing (Day/Month/Year)	Priority Claimed Under 35 USC 119
P1998-095448	**Japan**	**24 / March / 1998**	[X] Yes [ ] No
			[ ] Yes [ ] No

I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below.

PRIOR U.S. PROVISIONAL APPLICATIONS, BENEFIT CLAIMED UNDER 35 USC §119(e)

(Application No.)	(Filing Date)	(Application No.)	(Filing Date)
(Application No.)	(Filing Date)	(Application No.)	(Filing Date)

I hereby claim the benefit of Title 35, United States Code Section 120 of any United States application(s), or 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of Title 35, United States Code Section 112, I acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

PRIOR U.S. APPLICATIONS OR PCT INTERNATIONAL APPLICATIONS DESIGNATING THE U.S., BENEFIT CLAIMED UNDER 35 USC §120

PCT/JP99/01448	March 23, 1999	Pending
(Application No.)	(Filing Date)	(Status: Patented, Pending, Abandoned)
(Application No.)	(Filing Date)	(Status: Patented, Pending, Abandoned)

POWER OF ATTORNEY: As a named inventor, I hereby appoint the registered practitioners of Morgan, Lewis & Bockius LLP included in the Customer Number provided below to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Customer Number 009629

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WASHINGTON D.C., 20036-5869

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

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PATENT

ATTORNEY DOCKET NO. 46124-5042-US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	
	)	
Tadamitsu KISHIMOTO *et al.*	)	
	)	
Application No.:	)	Group Art Unit: Unassigned
(based on PCT/JP99/01448)	)	
	)	
Filed: March 23, 1999	)	Examiner: Unassigned
	)	
For: VASCULARIZATION INHIBITORS	)	

Commissioner for Patents
Washington, D.C. 20231
BOX SEQUENCE

STATEMENT ACCOMPANYING SEQUENCE LISTING

Dear Sir:

The undersigned hereby states upon information and belief that the Sequence Listing submitted concurrently herewith does not include matter which goes beyond the content of the application as filed and that the information recorded on the diskette submitted concurrently herewith is identical to the written Sequence Listing submitted herewith.

Respectfully submitted,
MORGAN, LEWIS & BOCKIUS LLP

Dated: 9.22.00 By: Rosanne Kosson

Printed Name: Rosanne Kosson
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SEQUENCE LISTING

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Lys Gln Gly Cys Glu Phe Glu Asn Thr Val His Lys Trp Ile Ser
275 280 285
Ile Thr Glu Ala Leu Ala Phe Phe His Cys Cys Leu Asn Pro Ile
290 295 300
Leu Tyr Ala Phe Leu Gly Ala Lys Phe Lys Thr Ser Ala Gln His
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Ser Ser Ser Phe His Ser Ser
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tcc atc atc ttc tta act ggc att gtg ggc aat gga ttg gtc atc 180
ctg gtc atg ggt tac cag aag aaa ctg aga agc atg acg gac aag 225
tac agg ctg cac ctg tca gtg gcc gac ctc ctc ttt gtc atc acg 270
ctt ccc ttc tgg gca gtt gat gcc gtg gca aac tgg tac ttt ggg 315
aac ttc cta tgc aag gca gtc cat gtc atc tac aca gtc aac ctc 360
tac agc agt gtc ctc atc ctg gcc ttc atc agt ctg gac cgc tac 405

ctg gcc atc gtc cac gcc acc aac agt cag agg cca agg aag ctg 450
ttg gct gaa aag gtg gtc tat gtt ggc gtc tgg atc cct gcc ctc 495
ctg ctg act att ccc gac ttc atc ttt gcc aac gtc agt gag gca 540
gat gac aga tat atc tgt gac cgc ttc tac ccc aat gac ttg tgg 585
gtg gtt gtg ttc cag ttt cag cac atc atg gtt ggc ctt atc ctg 630
cct ggt att gtc atc ctg tcc tgc tat tgc att atc atc tcc aag 675
ctg tca cac tcc aag ggc cac cag aag cgc aag gcc ctc aag acc 720
aca gtc atc ctc atc ctg gct ttc ttc gcc tgt tgg ctg cct tac 765
tac att ggg atc agc atc gac tcc ttc atc ctc ctg gaa atc atc 810
aag caa ggg tgt gag ttt gag aac act gtg cac aag tgg att tcc 855
atc acc gag gcc cta gct ttc ttc cac tgt tgt ctg aac ccc atc 900
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gca ctc acc tct gtg agc aga ggg tcc agc ctc aag atc ctc tcc 990
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Phe	Arg	Asp	Glu	Asn	Val	His	Phe	Asn	Arg	Ile	Phe	Leu	Pro	Thr	
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Ile	Tyr	Phe	Ile	Ile	Phe	Leu	Thr	Gly	Ile	Val	Gly	Asn	Gly	Leu	
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Ile	Thr	Leu	Pro	Phe	Trp	Ala	Val	Asp	Ala	Met	al.a	Asp	Trp	Tyr	
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Arg Tyr Leu Ala Ile Val His Ala Thr Asn Ser Gln Arg Pro Arg
140 145 150
Lys Leu Leu Ala Glu Lys Ala Val Tyr Val Gly Val Trp Ile Pro
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Ala Leu Leu Leu Thr Ile Pro Asp Phe Ile Phe Ala Asp Val Ser
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Gln Gly Asp Ile Ser Gln Gly Asp Asp Arg Tyr Ile Cys Asp Arg
185 190 195
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215 220 225
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230 235 240
Lys Arg Lys Ala Leu Lys Thr Thr Val Ile Leu Ile Leu Ala Phe
245 250 255
Phe Ala Cys Trp Leu Pro Tyr Tyr Val Gly Ile Ser Ile Asp Ser
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gac aag tac cgg ctg cac ctg tca gtg gct gac ctc ctc ttt gtc 270
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ttt ggg aaa ttt ttg tgt aag gct gtc cat atc atc tac act gtc 360
aac ctc tac agc agc gtt ctc atc ctg gcc ttc atc agc ctg gac 405
cgg tac ctc gcc att gtc cac gcc acc aac agt caa agg cca agg 450
aaa ctg ctg gct gaa aag gca gtc tat gtg ggc gtc tgg atc cca 495
gcc ctc ctc ctg act ata cct gac ttc atc ttt gcc gac gtc agc 540
cag ggg gac atc agt cag ggg gat gac agg tac atc tgt gac cgc 585
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His Leu Lys Ile Leu Asn Thr Pro Asn Cys Ala Leu Gln Ile Val
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Ala	Arg	Leu	Lys	Asn	Asn	Asn	Arg	Gln	Val	Cys	Ile	Asp	Pro	Lys	
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gca cgg ctg aag aac aac aac aga caa gtg tgc att gac ccg aaa 306
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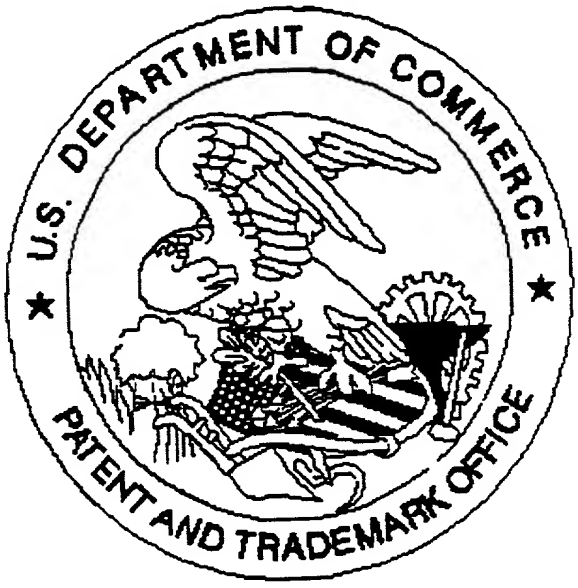
United States Patent & Trademark Office

Office of Initial Patent Examination -- Scanning Division



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